TOXICOLOGICAL SCIENCES **112(1)**, 153–163 (2009)
doi:10.1093/toxsci/kfp123
Advance Access publication June 5, 2009

# Identification of Chemical Compounds that Induce HIF-1α Activity

Menghang Xia,*[,1] Ruili Huang,* Yi Sun,† Gregg L. Semenza,‡ Shelley Force Aldred,§ Kristine L. Witt,¶ James Inglese,* Raymond R. Tice,¶ and Christopher P. Austin*

**NIH Chemical Genomics Center, National Institutes of Health, Bethesda, Maryland 20892; †Department of Radiation Oncology, University of Michigan, Comprehensive Cancer Center, Ann Arbor, Michigan 48109; ‡Vascular Biology Program, Institute for Cell Engineering, Departments of Pediatrics, Medicine, Oncology, Radiation Oncology, McKusick-Nathans Institute of Genetic Medicine, Johns Hopkins University School of Medicine, Baltimore, Maryland 21205; §SwitchGear Genomics, Menlo Park, California 94025; and ¶National Toxicology Program, National Institute of Environmental Health Sciences, Research Triangle Park, North Carolina 27709*

Received January 27, 2009; accepted May 29, 2009

**Cellular metabolism depends on the availability of oxygen and the major regulator of oxygen homeostasis is hypoxia-inducible factor 1 (HIF-1), a highly conserved transcription factor that plays an essential role in cellular and systemic homeostatic responses to hypoxia. HIF-1 is a heterodimeric transcription factor composed of hypoxia-inducible HIF-1α and constitutively expressed HIF-1β. Under hypoxic conditions, the two subunits dimerize, allowing translocation of the HIF-1 complex to the nucleus where it binds to hypoxia-response elements (HREs) and activates expression of target genes implicated in angiogenesis, cell growth, and survival. The HIF-1 pathway is essential to normal growth and development, and is involved in the pathophysiology of cancer, inflammation, and ischemia. Thus, there is considerable interest in identifying compounds that modulate the HIF-1 signaling pathway. To assess the ability of environmental chemicals to stimulate the HIF-1 signaling pathway, we screened a National Toxicology Program collection of 1408 compounds using a cell-based β-lactamase HRE reporter gene assay in a quantitative high-throughput screening (qHTS) format. Twelve active compounds were identified. These compounds were tested in a confirmatory assay for induction of vascular endothelial growth factor, a known hypoxia target gene, and confirmed compounds were further tested for their ability to mimic the effect of a reduced-oxygen environment on hypoxia-regulated promoter activity. Based on this testing strategy, three compounds (*o*-phenanthroline, iodochlorohydroxyquinoline, cobalt sulfate heptahydrate) were confirmed as hypoxia mimetics, whereas two compounds (7-diethylamino-4-methylcoumarin and 7,12-dimethylbenz(*a*)anthracence) were found to interact with HIF-1 in a manner different from hypoxia. These results demonstrate the effectiveness of qHTS in combination with secondary assays for identification of HIF-1α inducers and for distinguishing among inducers based on their pattern of activated hypoxic target genes. Identification of environmental compounds having HIF-1α activation activity in cell-based assays may be useful for prioritizing chemicals for further testing as hypoxia-response inducers *in vivo*.**

*Key Words:* **cobalt sulfate heptahydrate; 7-diethylamino-4-methylcoumarin; 7,12-dimethylbenz(*a*)anthracence; HIF-1α; inducers; iodochlorohydroxyquinoline; NTP 1408 compound library; *o*-phenanthroline; qHTS.**

Oxygen ($O_2$) levels play a critical role in governing many cellular pathways essential for mammalian cell survival. Hypoxia is defined as a reduction in the normal level of tissue oxygen tension. In response to hypoxia, mammalian cells activate hypoxia-inducible factor 1 (HIF-1), which regulates the transcription of genes involved in angiogenesis, erythropoiesis, glycolysis, iron metabolism, and cell survival (Mole and Ratcliffe, 2008; Semenza, 2001). HIF-1 is composed of two subunits: hypoxic responsive HIF-1α and constitutively expressed HIF-1β, also known as the aryl hydrocarbon receptor nuclear translocator (Wang and Semenza, 1995). Under normal oxidation conditions, HIF-1α is rapidly degraded by the ubiquitin-proteasome pathway (Huang *et al.*, 1998; Salceda and Caro, 1997). However, under hypoxic conditions (Fig. 1), intracellular HIF-1α is stabilized due to the attenuation of prolyl hydroxylase activity (Ivan *et al.*, 2001; Jaakkola *et al.*, 2001), which is required to initiate proteasomal degradation (Stolze *et al.*, 2006). The accumulated HIF-1α heterodimerizes with HIF-1β and translocates into the nucleus. The HIF-1 complex binds to DNA regulatory sequences known as hypoxia-response elements (HREs), which are present in the promoter or enhancer regions of HIF-1 target genes (Wenger *et al.*, 2005). After recruiting transcriptional coactivators such as p300 and the cAMP response element–binding protein (CBP) (Lando *et al.*, 2002), target gene expression is activated. To date, more than 70 hypoxia target genes have been identified (Wenger *et al.*, 2005), including the vascular endothelial growth factor (VEGF) (Forsythe *et al.*, 1996). Other well-known target genes include aldolase A and lactate dehydrogenase A, both involved in glycolysis (Firth *et al.*, 1995; Semenza *et al.*, 1996), and the iron metabolism regulator

[1] To whom correspondence should be addressed at 9800 Medical Center Drive, NIH Chemical Genomics Center, National Institutes of Health, Bethesda, MD 20892-3370. Fax: (301) 217-5736. E-mail: mxia@mail.nih.gov.

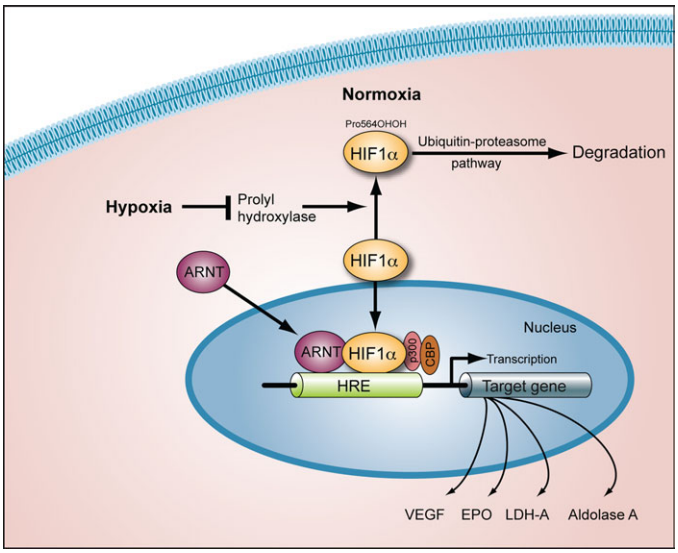


**FIG. 1.** During normoxia, HIF-1α is rapidly degraded by the ubiquitin-proteasome pathway. However, under hypoxic conditions, prolyl hydroxylase activity is attenuated, which decreases the proteasomal degradation of intracellular HIF-1α. The accumulated HIF-1α heterodimerizes with HIF-1β and translocates into the nucleus. The HIF-1 complex binds to DNA regulatory sequences known as HREs, which are present in the promoter or enhancer regions of HIF-1 target genes. After recruiting transcriptional coactivators, such as p300 and CBP, the expression of target genes including VEGF, EPO, and LDH-A, are initiated. Abbreviations: ARNT = aryl hydrocarbon receptor nuclear translocator; EPO = erythropoietin; LDH = lactate dehydrogenase.

transferrin receptor (Mukhopadhyay *et al.*, 2000; Tacchini *et al.*, 1999).

The importance of HIF-1 to normal growth and development is well established, as is its role in the control of energy metabolism, angiogenesis, and erythropoiesis; this pathway is involved also in the pathophysiology of cancer, inflammation, and ischemia (Dery *et al.*, 2005; Maxwell, 2005; Mole and Ratcliffe, 2008; Maxwell and Salnikow, 2004; Maxwell *et al.*, 2001; Ryan *et al.*, 1998; Semenza, 2003). Thus, inhibitors of this pathway are of interest for their ability to potentially be used as anti-tumor agents (Shannon *et al.*, 2003), whereas inducers may affect normal development and/or aggravate disease progression. Recently, using a cell-based pathway assay (β-lactamase HRE reporter gene assay), we identified several small molecular compounds that inhibit the HIF-1 signaling pathway (Xia, Bi, Huang, Cho, Sakamuru, Miller, Printen, Austin, and Inglese, unpublished data). While conducting this study, it occurred to us that it would also be of interest to screen for compounds to which humans might be exposed that are capable of inducing HIF-1. For this purpose, we have screened a collection of 1408 compounds (1353 unique, 55 duplicate) provided by the National Toxicology Program (NTP) to the National Institutes of Health (NIH) Chemical Genomics Center (NCGC) as part of their high-throughput screening initiative (http://ntp.niehs.nih.gov/go/28213) within NTP's Vision and Roadmap for moving toxicology from a predominantly observational science at the level of disease-specific models to a predominantly predictive science focused upon a broad inclusion of target-specific, mechanism-based, biological observations (http://ntp.niehs.nih.gov/go/vision). These compounds were selected for this initial library because they represented a diverse chemical space and almost all had been tested by NTP in one or more standard toxicological tests. Inducers of HIF-1 were detected using a cell-based β-lactamase HRE reporter gene assay in a quantitative high-throughput screening (qHTS) format (Xia *et al.*, 2008; Xia *et al.*, 2009). Compounds identified as active in the qHTS screen were studied in secondary assays that measured VEGF secretion and hypoxia-responsive transcriptional regulatory element activity. Using this comprehensive approach, we identified several inducers of the hypoxia-signaling pathway among the 1408 NTP compounds. The results indicate that this approach may be broadly useful in identifying other substances that induce this pathway and for distinguishing among HIF-1 inducers based on patterns of activated hypoxic target genes.

## MATERIALS AND METHODS

***Cell lines and culture conditions.*** CellSensor HRE-*bla* ME-180 cells (HRE-*bla* cells), which stably express a β-lactamase (*bla*) reporter gene under the control of an HRE, were obtained from Invitrogen (Carlsbad, CA). The ME-180 cell line originates from human cervical cancer cells (Sykes *et al.*, 1970). All of the cell culture reagents were obtained from Invitrogen. HRE-*bla* cells were cultured in Dulbecco's Modified Eagle Media (DMEM) medium supplemented with 10% dialyzed fetal bovine serum (FBS), 2mM L-glutamine, 0.1mM nonessential amino acids, 1mM sodium pyruvate, 25mM N-2-hydroxyethylpiperazine-N'-2-ethane sulfonic acid, 50 U/ml penicillin, 50 μg/ml streptomycin, and 5 μg/ml of blasticidin. Mouse embryo fibroblast (MEF) cell lines from HIF-1$\alpha^{+/+}$ or HIF-1$\alpha^{-/-}$ mice were immortalized by transfection with plasmid pOT, which contains the SV40 early region encoding T antigen (Feldser *et al.*, 1999). These cells were cultured in DMEM medium supplemented with 15% FBS and 0.1mM nonessential amino acids. All cells were maintained at 37 ± 1°C under a humidified atmosphere and 5% $CO_2$.

***Compounds.*** In support of the NTP high-throughput screening initiative, the NTP provided an initial library of 1408 compounds (1353 unique compounds, 55 compounds in duplicate to evaluate assay reproducibility) to the NCGC. The compounds were dissolved in dimethyl sulfoxide (DMSO, Fisher Scientific, Pittsburgh, PA) at a stock concentration of 10mM. Selection of compounds for this initial library was based largely on the availability of historical *in vitro* and/or *in vivo* toxicological data from studies conducted by the NTP, as well as solubility in DMSO at 10mM and lack of excessive volatility. A list of the compounds in this library is provided at (http://www.ncbi.nlm.nih.gov/sites/entrez?db = pcsubstance&term = NTPHTS).

For more extensive testing, 13 compounds were purchased from Sigma-Aldrich (St Louis, MO). These compounds are 2-aminoanthracene (Chemical Abstracts Services Registry Number [CASRN] = 613-13-8; purity = 96%); benzo(*b*)fluoranthene (CASRN = 205-99-2, purity = 98%); benzo(*k*)fluoranthene (CASRN = 207-08-9, purity = 98%), cobalt sulfate ($CoSO_4 7H_2O$; CASRN = 10026-24-1; purity = 99%); dibenz(*a*,*h*)anthracene (CASRN = 53-70-3; purity = 97%); 7-diethylamino-4-methylcoumarin (7-DEA-4-MC; CASRN = 91-44-1; purity = 99%); 7,12-dimethylbenz(*a*)anthracence (7,12-DMBA; CASRN = 57-97-6; purity = 95%); iodochlorohydroxyquinoline (CASRN = 130-26-7; purity = 95%); *o*-phenanthroline (CASRN = 66-71-7; purity = 99%); prednisone (CASRN = 53-03-2; purity = 98%);

**TABLE 1**
**qHTS Protocol**

| Step | parameter | Value | Description |
|---|---|---|---|
| 1 | Plate cells | 5 μl | 2500 HRE-*bla* cells |
| 2 | Incubation time | 4–6 h | Cells adhere and acclimate |
| 3 | Library compound | 23 nl | 92μM to 0.59nM titration series |
| | Positive control compound ($CoCl_2$) | 23 nl | 0.91–400μM titrations |
| 4 | Incubation time | 17 h | Induce HRE reporter |
| 5 | Reagent | 1 μl | Beta lactamase detection mix |
| 6 | Incubation time | 2.5 h | Cells load and cleave dye |
| 7 | Assay Readout | Ex = 405/8 nm | Envision |

*Notes*.

1. Black clear bottom 1536-well plates, single tip dispense of 2500 cells per well into all wells.
2. 37 ± 1°C under a humidified atmosphere and 5% $CO_2$ incubator.
3. Pintool transfer of library to columns 5–48, and controls to columns 1–4.
4. 37 ± 1°C under a humidified atmosphere and 5% $CO_2$ incubator.
5. Single tip dispense the mixture provided by Invitrogen of 0.6μM CCF4-AM, 1 mg/ml Pluronic F127 surfactant, 3.5% PEG 400, 2.6% TR40, 2mM probenecid, 0.1% dimethyl sulfoxide.
6. Room temperature.
7. 460/25 and 530/20-nm emission filters and 405/8 excitation filter.

salicylazosulfapyridine (CASRN = 599-79-1; purity = 98%); and triamterene (CASRN = 396-01-0; purity = 99%). Cobalt chloride ($CoCl_27H_2O$, CASRN = 7791-13-1; purity = 99%), the positive control compound for these studies, was also purchased from Sigma-Aldrich.

***HRE β-lactamase reporter gene assay and qHTS.*** The general assay protocol is described in Table 1. Briefly, HRE-*bla* cells were suspended in OPTI-MEM medium (reduced-serum medium, Invitrogen) containing 0.5% dialyzed FBS and were dispensed at 2500 cells/5 μl/well in 1536-well black wall/clear bottom plates (Kalypsys, San Diego, CA) using a Flying Reagent Dispenser (FRD, Aurora Discovery, Carlsbad, CA). After the cells were incubated overnight at 37 ± 1°C under a humidified atmosphere and 5% $CO_2$, 23 nl of each compound in the NTP 1,408 compound library dissolved in DMSO at a concentration of 10mM was transferred to the assay plate by a pintool (Kalypsys), resulting in final compound concentrations of 0.59nM to 46μM, and 0.45% DMSO. To achieve a final compound concentration of 92μM (DMSO concentration 0.9%), 23 nl was transferred twice from the highest concentration mother plate into each well of the assay plate; control plates using DMSO only at this higher concentration were included also. Therefore, the final compound concentrations in the 5-μl assay volume ranged from 0.59nM to 92μM in 14 concentrations (Xia *et al*., 2008). The total number of plates was 18, including four DMSO-only plates (two at each final DMSO concentration of 0.45 and 0.9%). Each treatment plate included concurrent DMSO and positive control wells; the positive control was $CoCl_2$, a known chemical hypoxic mimetic (Maxwell and Salnikow, 2004). The controls were arrayed as follows: Column 1, concentration-response titration of $CoCl_2$ from 0.91 to 400μM; Column 2, 100μM $CoCl_2$; Column 3, DMSO only; and Column 4, 60μM $CoCl_2$. The concentration-response titration for $CoCl_2$ was used to evaluate plate-to-plate consistency, based on the calculated effective concentration that induced a half-maximal response (i.e., the $EC_{50}$), whereas the DMSO control and 100μM $CoCl_2$ data were used to normalize the test compound data on each plate. The 60μM $CoCl_2$ concentration was included in the event the 100μM $CoCl_2$ proved to be cytotoxic. The plates were incubated for 17 h at 37 ± 1°C under a humidified atmosphere and 5% $CO_2$; this sample time is optimal for this assay (Xia *et al*., unpublished data). Evaluation of HIF-1 response was determined according to manufacturer's instructions. Briefly, after 1 μl of LiveBLAzer B/G FRET substrate (Invitrogen, CA) was added, the plates were incubated at room temperature for 2–2.5 h, and fluorescence intensity at 460 and 530 nm emission was measured at 405 nm excitation by an Envision plate reader (Perkin Elmer, Shelton, CT). Data were expressed for each wavelength separately and as the ratio of 460 nm/530 nm emissions.

To confirm the results of the initial study, compounds classified as active in this assay (see section on HRE β-lactamase reporter gene assay data analysis) were retested in the HRE-*bla* assay. The assay protocol was the same as described above except the concentration titrations were all within one 1536-well plate and the compounds were tested at 16 concentrations in quadruplicate.

***HRE β-lactamase reporter gene assay data analysis.*** Primary data analysis was performed as previously described (Inglese *et al*., 2006; Xia *et al*., 2009). Briefly, raw plate reads for each titration point were first normalized relative to the 100μM $CoCl_2$ positive control response (i.e., 100% response) and the basal response in the DMSO-only wells (i.e., 0% response), and then corrected by applying a pattern correction algorithm using the compound-free DMSO control plates. Concentration-response titration points for each compound were fitted to a four-parameter Hill equation yielding concentrations of half-maximal activity ($EC_{50}$) and maximal response (efficacy) values. The concentration-response curves were sorted into four major classes (1–4) using previously published criteria (Inglese *et al*., 2006; Xia *et al*., 2009). Curve classes were further subdivided to provide more detailed classification. Briefly, we have the greatest confidence that compounds with class 1.1, 1.2, and 2.1 curves are true actives, and less confidence in compounds with class 1.3, 1.4, 2.2, and 3 curves. Curve class 4 compounds showed no concentration response and are defined as inactive compounds. Cytotoxic or fluorescent compounds may interfere with the ratio reading in a HRE *bla* assay. Therefore, we examined all three readings (i.e., 530 nm, 460 nm, and the ratio) when considering compounds for follow-up studies. Fifteen compounds had class 1–3 curves in both the 460 nm and the ratio readings, but three of these also exhibited a significant decrease in the 530-nm reading, an indication of potential cytotoxicity over the concentration range tested. These compounds were not evaluated further. Two of the 12 compounds showed an increase in the 530-nm reading, which might indicate potential auto-fluorescence. However, due to the small number of compounds that met the first set of criteria, we selected all 12 compounds for follow-up testing (Fig. 2). In addition, we included $CoCl_2$, the positive control compound in the primary screen, in the follow-up studies as an internal reference compound.

***Measurement of VEGF secretion.*** ME-180 cells were plated in growth medium at 1 x $10^5$ cells per well in a 24-well plate. MEF cells from HIF-$1\alpha^{+/+}$ or HIF-$1\alpha^{-/-}$ mice were plated in growth medium at 2.5 x $10^4$ cells per well in a 96-well plate. After incubation for 3–5 h at 37 ± 1°C under a humidified atmosphere and 5% $CO_2$, the cell culture medium was removed and OPTI-MEM medium with 1% dialyzed FBS for HRE-*bla* cells and 15% FBS for MEF cells was added to the wells. The cells were then treated with compounds at 8 concentrations of 0.1–200μM at 37 ± 1°C under a humidified atmosphere and 5% $CO_2$. A maximum concentration of 200μM was chosen in the assay to cover wider range of compound concentrations. After 20 h of treatment, the culture medium was removed and analyzed for VEGF expression using a human or mouse VEGF immunoassay kit (R&D Systems, Minneapolis, MN). Briefly, for the human VEGF protocol, 200 μl of sample or human VEGF standard (0–2000 pg/ml) was added to wells of a microplate precoated with a human monoclonal antibody specific to VEGF. For the mouse VEGF protocol, 50 μl of sample or mouse VEGF standard (0–500 pg/ml) was added to wells of a microplate precoated with a mouse monoclonal antibody specific to VEGF. The plates were incubated at room temperature for 2 h. After washing away any unbound substances, an anti-VEGF antibody conjugated to horseradish peroxidase was added and the plate incubated for 2 h at room temperature. Following three to five washes, a substrate solution was added and incubated for 20–30 min, followed by the addition of a stop solution. The optical density of each well was determined using an EnVision plate reader at 450 nm with 570 nm as a reference filter. The reading from each sample was

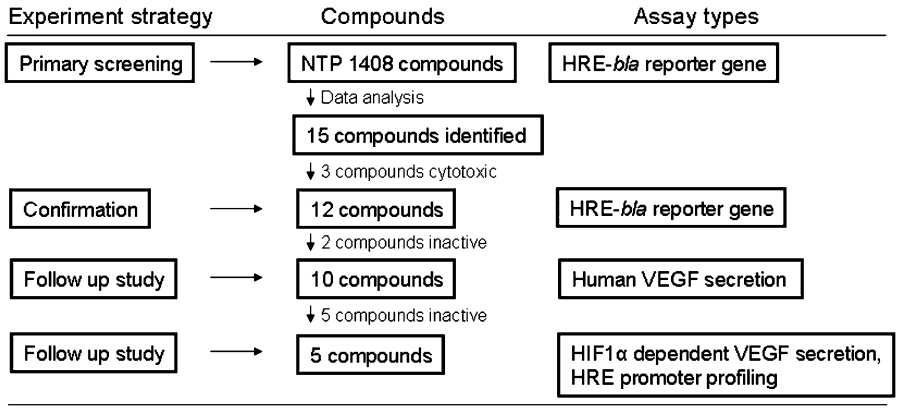


**FIG. 2.** After primary qHTS, 15 compounds were identified based on data analysis. Three of 15 compounds (crystal violet lactone, mercuric chloride, 9-aminoacridine) exhibited significant cytotoxicity and were therefore excluded from the follow-up study. Twelve compounds listed in the method were tested in HRE-*bla* assay. Ten confirmed compounds in HRE-*bla* assay were tested in a human VEGF secretion assay. Only five compounds—7 diethylamino-4-methylcoumarin, 7,12-dimethylbenz(*a*)anthracence, iodochlorohydroxyquinoline, $CoSO_4$, and *o*-phenanthroline—as well as $CoCl_2$, the positive control, showed positive response in the human VEGF secretion assay. These five compounds were further tested in HIF1α dependent VEGF secretion assay and for HRE promoter profiling.

obtained from 450 nm after subtracted the reading at 570 nm, and then the VEGF levels were calculated based on the VEGF standard. The concentration-response curve of each compound was fitted by Prism GraphPad (GraphPad Software Inc., La Jolla, CA). All the compounds with a curve fit were defined as positive. The results presented are based on three independent experiments.

***Measurement of hypoxia-responsive transcriptional regulatory elements.*** The effect of selected chemical compounds on 36 human hypoxia-regulated promoters and long-range transcriptional regulatory elements (LREs) was measured using reporter constructs from SwitchGear Genomics (Menlo Park, CA) (see Table 2). Briefly, promoter-reporter vectors were constructed by cloning ~1-kb promoter fragments from 34 known and candidate hypoxia-regulated genes into a multiple cloning site upstream of the firefly luciferase (*luc2P*) reporter cassette from Promega (Madison, WI). In addition, LRE fragments from the VEGF (~1100 bp) and erythropoietin (EPO; ~600 bp) loci were cloned upstream of a basal HSV-TK promoter in the same *luc2P* reporter vector. Two sets of control promoter vectors were used to adjust for subtle plate-to-plate signal variation (four constitutive promoters, four random genomic fragments) and for overall signal variation based on the nonspecific response of cells to different treatments (six randomly chosen promoters not specifically known to respond to hypoxia, six additional random genomic fragments).

Transient transfection assays were conducted in HCT116 human colon carcinoma cells (ATCC, Manassas, VA) in 96-well plates. Per well, 7500 cells were seeded in culture medium. Twenty-four hr later, 50 ng of plasmid DNA were added per well and then transfected with Fugene-6 transfection reagent (Roche Diagnostics, Indianapolis, IN), according to Fugene standard protocols. After 20 h, the transfection medium was removed and fresh culture medium was added into each well. The transfected cells were treated with or without chemical compounds or $1\%O_2/5\%CO_2/94\%\ N_2$, a level of oxygen that induces hypoxia in cultured cells (Chau *et al*., 2005), for 20 h. The final concentrations of *o*-phenanthroline, iodochlorohydroxyquinoline, $CoCl_2$, 7-DEA-4-MC, 7,12-DMBA, and $CoSO_4$ were 10, 15, 100, 100, 100, and 100μM, respectively. These concentrations represented approximately the $EC_{80}$ values determined in the VEGF secretion assay. At the end of the treatment period, 100 μl of Steady-Glo (Promega) was added to each well and the plates were incubated at room temperature for 30 min. After 30 min, the plates were read in a standard plate luminometer (Molecular Devices, Sunnyvale, CA). Each compound was assayed in three wells.

After normalizing for plate-to-plate and between-condition signal variation, a two-tailed Student's *t*-test ($p < 0.05$) was used to assess whether the activity of the fragment was different in the untreated control condition compared with each of the treated conditions. Parametric statistics were used based on historical data for this assay. For those responses that were significantly different, a log2 ratio of activity was calculated in treated/untreated conditions.

Hierarchical clustering of the log2 induction ratios was performed using the Xcluster engine described at http://fafner.stanford.edu/~sherlock/cluster.html. Both the genes and conditions were clustered using the Pearson correlation as the centering metric. A heat map of the clustered data was generated using Java TreeView, which is described in more detail at http://jtreeview.sourceforge.net/. Similarity between the compound-exposed gene expression profile and the expression profile under low oxygen was assessed by calculating the Pearson correlation coefficient (*R*).

***Cell viability assay.*** Cell viability was measured using a luciferase-coupled adenosine triphosphate (ATP) quantitation assay (CellTiter-Glo, Promega). HRE-*bla* cells were dispensed at 2500 cells/well in 1536-well white/solid bottom assay plates (Greiner Bio-One North America, Monroe, NC) using a FRD. The cells were incubated for 4–6 h at 37 ± 1°C under a humidified atmosphere and 5% $CO_2$ to allow for cell attachment, followed by addition of compounds via pintool. After compound addition, plates were again incubated for 17 h also at 37 ± 1°C under a humidified atmosphere and 5% $CO_2$. At the end of the incubation period, 5 μl of CellTiter-Glo reagent was added, plates were incubated at room temperature for 30 min, and luminescence intensity was determined using a ViewLux plate reader (PerkinElmer; Shelton, CT). Data were analyzed as described previously (Xia *et al*., 2008). Briefly, data were normalized and corrected using the DMSO controls and positive control compound (Tamoxifen), and concentration-response curves were fit to the Hill equation and classified as described in the HRE β-lactamase assay data analysis section. Compounds with class 1.1, 1.2, or 2.1 curves were considered cytotoxic, compounds with class four curves were inactive or not cytotoxic, and other curve classes were considered inconclusive.

## RESULTS

### *Identification of Chemical Inducers of HIF-1 Signaling Pathway Using qHTS*

To identify chemical inducers of HIF-1α activity, we used an HRE β-lactamase (HRE-*bla*) reporter gene assay to screen

**TABLE 2**
**Sequence Coordinates of Transcriptional Regulatory Elements Cloned into a Luciferase-based Reporter Vector**

| Gene symbol | Regulatory element type | Chromosome | Strand | Start | Stop |
|---|---|---|---|---|---|
| EGLN1 | Promoter | chr1 | − | 227864603 | 227865538 |
| SERPINE1 | Promoter | chr7 | + | 100362990 | 100363963 |
| ALDOC | Promoter | chr17 | − | 23927908 | 23928910 |
| ENO2 | Promoter | chr12 | + | 6893278 | 6894187 |
| PDK3 | Promoter | chrX | + | 24242350 | 24243334 |
| MIF | Promoter | chr22 | + | 22560193 | 22561234 |
| GAPDH | Promoter | chr12 | + | 6513163 | 6514226 |
| ANKRD37 | Promoter | chr4 | + | 186692196 | 186693210 |
| ALDOA | Promoter | chr16 | + | 29983757 | 29984753 |
| PDK1 | Promoter | chr2 | + | 173245436 | 173246536 |
| ENO1 | Promoter | chr1 | − | 8872899 | 8873965 |
| NPPB | Promoter | chr1 | − | 11853091 | 11854107 |
| PGK1 | Promoter | chrX | + | 77165228 | 77166132 |
| TFRC | Promoter | chr3 | − | 197297200 | 197298143 |
| EPO | Long-range element | chr7 | + | 99965917 | 99966536 |
| MDM2 | Promoter | chr12 | + | 67487240 | 67488306 |
| LDHA | Promoter | chr11 | + | 18372059 | 18372891 |
| HK2 | Promoter | chr2 | + | 74971865 | 74973064 |
| HK1 | Promoter | chr10 | + | 70747674 | 70748753 |
| VEGFA | Long-range element | chr6 | + | 43844373 | 43845510 |
| P4HA2 | Promoter | chr5 | − | 131591338 | 131592267 |
| EGR1 | Promoter | chr5 | + | 137828216 | 137829238 |
| HIG2 | Promoter | chr7 | + | 127688963 | 127689998 |
| CA9 | Promoter | chr9 | + | 35663134 | 35664055 |
| CAT | Promoter | chr11 | + | 34416135 | 34417129 |
| PFKM | Promoter | chr12 | + | 46798509 | 46799467 |
| PPP2R1B | Promoter | chr11 | − | 111142221 | 111143302 |
| IL1R1 | Promoter | chr2 | + | 102227910 | 102228989 |
| EGLN2 | Promoter | chr19 | + | 45995942 | 45997017 |
| HIF3A | Promoter | chr19 | + | 51491197 | 51492271 |
| AK1 | Promoter | chr9 | − | 127719449 | 127720490 |
| ERCC2 | Promoter | chr19 | − | 50565472 | 50566258 |
| GPX3 | Promoter | chr5 | + | 150379483 | 150380488 |
| HSP90AB2P | Promoter | chr4 | + | 13010460 | 13011484 |
| ACE | Promoter | chr17 | + | 58914950 | 58916020 |
| BNIP3L | Promoter | chr8 | + | 26302921 | 26303893 |

*Note*. Coordinate system: UCSC Genome Browser hg17/May 2004 Freeze (genome.ucsc.edu).

a NTP set of 1408 compounds (1353 unique compounds) at 14 concentrations ranging from 0.59nM to 92μM. To monitor plate-to-plate variations during the qHTS process, the concentration titration of $CoCl_2$, the positive control compound, was carried out in each assay plate. The $CoCl_2$ concentration-response curves reproduced well in all 18 plates with an average $EC_{50}$ value and standard deviation (SD) of 45 ± 5μM. The average and SD signal to background ratio for $CoCl_2$ was 7.3 ± 0.4; and the average $Z'$ factor and SD was 0.69 ± 0.07 from the 18 plates. These results demonstrate the robust nature of the β-lactamase reporter gene assay.

After primary qHTS, the concentration-response curves of the 1408 compounds were classified into four major curve classes (1–4; see Methods for details). Compounds that showed activation (nonclass 4) in both the ratio and the 460 nm channel were considered potentially positive compounds. Fifteen such compounds were identified (Fig. 2). Among these 15, three (crystal violet lactone, mercuric chloride, and 9-aminoacridine) also showed a significant decrease in signal in the 530 nm channel, an indication of cytotoxicity (Supplementary Fig. 1) over the concentration range tested. We therefore excluded these three compounds and selected the remaining twelve compounds for a confirmation study (Fig. 2, Table 3). Potencies ($EC_{50}$) and efficacies of active compounds were derived from the curve fits, with $EC_{50}$ values ranging from 7.9 to 50μM. Included among the 12 compounds was $CoSO_4$ ($EC_{50}$ = 31μM), another salt form of the positive control hypoxia-response inducer $CoCl_2$.

### *Confirmation of HIF-1α Inducers*

Twelve compounds identified from the qHTS as potential inducers of HIF-1 were retested in the HRE-*bla* assay. Ten of the twelve compounds showed similar activity in the confirmation study as in the primary screen, and two did not (Table 3). Of the 10 confirmed compounds, *o*-phenanthroline was the most potent, with an $EC_{50}$ of 7.9μM in the primary qHTS and 8.2μM in the validation assay. The rank order of potency of the ten compounds in the confirmation study was: *o*-phenanthroline, $CoSO_4$, prednisone, 2-aminoanthracene, iodochlorohydroxyquinoline, dibenz(*a*,*h*)anthracene, benzo(*b*)fluoranthene, 7-DEA-4-MC, triamterene, and 7,12-DMBA (Table 3). Efficacy was > 70% of control ($CoCl_2$) for nine out of the ten compounds, with iodochlorohydroxyquinoline showing 27% efficacy (Table 3). The concentration-response curves of these compounds are provided in Supplementary Figure 2.

Cytotoxicity of these compounds was additionally investigated in a cell viability assay that measures intracellular ATP content. Only iodochlorohydroxyquinoline and *o*-phenanthroline showed significant cytotoxicity, with $IC_{50}$ (calculated inhibitory concentration that induced a half-maximal response) values of 8 and 18μM, respectively. The relatively low efficacy of iodochlorohydroxyquinoline in the qHTS HRE-*bla* assay may be due to these cytotoxic effects, which would attenuate the observable reporter gene activation. None of the other compounds showed significant cytotoxicity over the concentration range tested.

### *Effect of HIF-1α Inducers on VEGF Secretion*

Hypoxia induces expression of VEGF (Forsythe *et al.*, 1996), one of the well-studied HIF-1 target genes (Chiarugi *et al.*, 1999; Forsythe *et al.*, 1996). To investigate whether the induction of HIF-1α signaling by these ten compounds results in an increase in VEGF secretion, we measured VEGF levels in ME-180 human cervical cancer cells following compound

**TABLE 3**
**Potencies (μM) and Efficacies (%) of HIF-1 Inducers Identified from qHTS and Confirmation Study**

| CASRN | Name | $EC_{50}$ (qHTS) | $EC_{50}$ (confirmation) | Efficacy (%) |
|---|---|---|---|---|
| 613-13-8 | 2-Aminoanthracene | 39.8 | 29.4 ± 5.3 | 52 ± 19 |
| 205-99-2 | Benzo(*b*)fluoranthene | 39.8 | 38.3 ± 13.1 | 143.5 ± 67 |
| 207-08-9 | Benzo(*k*)fluoranthene | 39.8 | Inactive | |
| 10026-24-1 | $CoSO_4$ | 31.6 | 11.0 ± 1.8 | 69 ± 6 |
| 53-70-3 | Dibenz(*a*,*h*)anthracene | 39.8 | 31.0 ± 4.6 | 127 ± 21.2 |
| 91-44-1 | 7-Diethylamino-4-methylcoumarin | 50.1 | 38.8 ± 2.9 | 75 ± 17 |
| 57-97-6 | 7,12-Dimethylbenz(*a*)anthracence | 39.8 | 47.5 ± 4.8 | 73 ± 9 |
| 130-26-7 | Iodochlorohydroxyquinoline | 12.6 | 29.8 ± 7.3 | 27 ± 6 |
| 66-71-7 | *o*-Phenanthroline | 7.9 | 8.2 ± 0.7 | 75 ± 20 |
| 53-03-2 | Prednisone | 31.6 | 12.4 ± 2.1 | 172 ± 13 |
| 599-79-1 | Salicylazosulfapyridine | 0.3 | Inactive | |
| 396-01-0 | Triamterene | 50.1 | 38.8 ± 2.9 | 60.0 ± 11.4 |

*Note*. Abbreviations: Efficacy = maximal response as percentage of the $CoCl_2$ in the confirmation study.
In the confirmation study, each value is the mean plus or minus SD of the results of four replicates.

treatment. As shown in Figure 3, only five compounds significantly induced VEGF secretion in a concentration-dependent manner. The mean rank order of potency (based on $EC_{50}$ values) calculated from three experiments in the VEGF assay was *o*-phenanthroline (3.1μM), iodochlorohydroxyquinoline (5.7μM), 7,12-DMBA (31.2μM), 7-DEA-4-MC (41.0μM), and $CoSO_4$ (50.1μM). The positive control, $CoCl_2$ ($EC_{50}$, 45μM), also induced VEGF secretion with a response pattern similar to the titration curve of $CoSO_4$. Five compounds (prednisone, 2-aminoanthracene, dibenz(*a*,*h*)anthracene, benzo(*b*)fluoranthene, triamterene) did not stimulate VEGF secretion in this assay even though they were shown to induce HIF-1α activity in the HRE-*bla* assay.

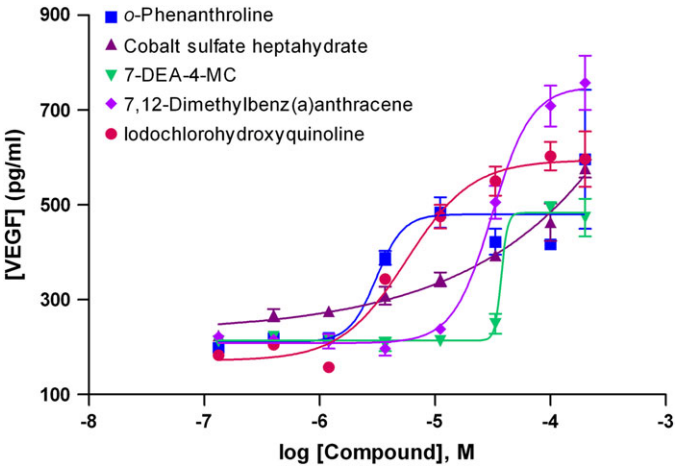


**FIG. 3.** Effect of compounds on VEGF secretion in ME-180 cells. After cells were treated with various concentrations of *o*-phenanthroline (–■–), $CoSO_4$ (–▲–), 7-DEA-4-MC (7-diethylamino-4-methylcoumarin; –▼–), 7,12-dimethylbenz(*a*)anthracence (–◆–), or iodochlorohydroxyquinoline (–●–) for 20 h, VEGF levels in the cell supernatant were measured. Each concentration-response curve represents the average response from three independent experiments while error bars represent the SD.

### *HIF-1α Dependence of Hypoxia-Signaling Induction*

To further investigate the relationship between VEGF and HIF-1α signaling by the five VEGF-inducers, VEGF secretion from MEF cells obtained from HIF-$1\alpha^{+/+}$ or HIF-$1\alpha^{-/-}$ mice (Feldser *et al.*, 1999; Tan *et al.*, 2008) was measured. As shown in Figure 4, *o*-phenanthroline (Fig. 4A), iodochlorohydroxyquinoline (Fig. 4B), and $CoSO_4$ (Fig. 4C) stimulated VEGF secretion in a concentration-dependent manner in HIF-$1\alpha^{+/+}$ MEF cells but had no or minimal effect on VEGF secretion in HIF-$1\alpha^{-/-}$ MEF cells. $CoCl_2$, the positive control in this study showed, a same response pattern as $CoSO_4$ (data not shown). Among these three compounds, *o*-phenanthroline was the most potent ($EC_{50}$ = 7.5μM), and the response observed with *o*-phenanthroline in the MEF knockout cells was similar to what was observed in ME-180 cells ($EC_{50}$ = 3.1μM). Iodochlorohydroxyquinoline was less potent in HIF-$1\alpha^{+/+}$ MEF cells ($EC_{50}$ = 31.6μM) than in ME-180 cells ($EC_{50}$ = 5.7μM). In contrast, 7-DEA-4-MC (Fig. 4D) did not stimulate VEGF in either cell type, and treatment with 7,12-DMBA (Fig. 4E) resulted in weak stimulation of VEGF secretion in both HIF-$1\alpha^{-/-}$ and HIF-$1\alpha^{+/+}$ MEF cells. These results indicate that VEGF secretion induced by *o*-phenanthroline, iodochlorohydroxyquinoline, and $CoSO_4$ is HIF-1α dependent, whereas VEGF secretion induced by 7,12-DMBA appears to be independent of HIF-1α. As opposed to its ability to induce VEGF in ME-180 cells, 7-DEA-4-MC did not upregulate VEGF in MEF cells; the basis for this difference is unknown.

### *Effect of Chemical Inducers on Hypoxia-Responsive Transcriptional Regulatory Elements: Promoter Profiling*

More than 70 hypoxia-regulated genes have been identified (Rocha, 2007; Wenger *et al.*, 2005). Assessment of the effects of compounds on the regulation of these genes may yield valuable insight into subtle differences in the way they affect other regulatory elements. To profile the effects of the five

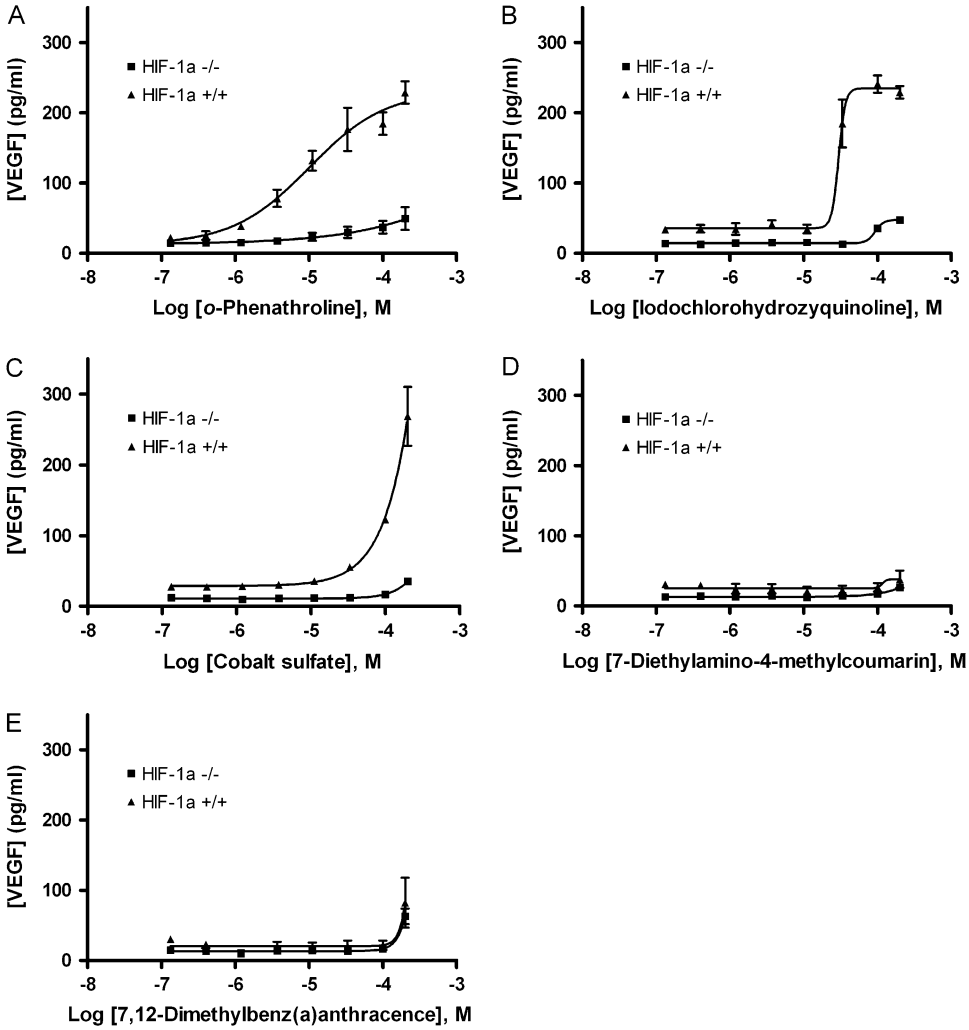


**FIG. 4.** HIF-1α dependent VEGF secretion induced by potential hypoxia inducers. Embryonic fibroblasts from HIF-$1\alpha^{+/+}$ or HIF-$1\alpha^{-/-}$ mice were plated in 96-well plate and incubated with various concentrations of *o*-phenanthroline (A), iodochlorohydroxyquinoline (B), $CoSO_4$ (C), 7-DEA-4-MC (7-diethylamino-4-methylcoumarin (D), and 7,12-dimethylbenz(*a*)anthracence (E) for 20 h. The VEGF levels in the cell supernatant were measured. Each concentration-response curve represents the average response from three independent experiments while error bars represent the SD.

VEGF-inducing compounds (plus the positive control, $CoCl_2$) on hypoxia-responsive promoters and LREs from the human genome, we selected 36 known and candidate response elements to test in a luciferase reporter-based system (Table 2). The elements were chosen based on HRE motif content, expression data, and previous hypoxia reporter experiments (SwitchGear Genomics, unpublished data). *o*-Phenanthroline, $CoSO_4$, and the positive control, $CoCl_2$, generated promoter activity profiles very similar to those produced by 1% $O_2$ (the standard hypoxic condition for *in vitro* studies), with correlation coefficients ($R$) of 0.96, 0.92, and 0.92, respectively (Table 4). The promoter activity profile produced by iodochlorohydroxyquinoline had a slightly lower but still statistically significant correlation with 1% $O_2$ ($R = 0.46$, $p < 0.05$). In contrast, the activity profiles for 7-DEA-4-MC and 7,12-DMBA (Table 4) were not correlated with 1% $O_2$ ($p > 0.05$). These results suggest that *o*-phenanthroline, iodochlorohydroxyquinoline, $CoSO_4$, and $CoCl_2$ induce the same hypoxia-responsive promoter activities as hypoxia. The lack of a significant correlation for 7-DEA-4-MC and 7,12-DMBA is consistent with the finding that these two compounds, in contrast to the other four HIF-1 inducing compounds, did not induce VEGF in MEF cells. The detailed information of induction data, such as log2 ratios of treated to untreated signals from all of the cloned fragments is listed in Supplementary Table 1.

As shown in Figure 5, 1% $O_2$, *o*-phenanthroline, $CoSO_4$, and $CoCl_2$ activated many HREs including those for the prolyl hydroxylase domain-containing protein (*EGLN1*), aldolase C (*ALDOC*), enolase 2 (*ENO2*), pyruvate dehydrogenase kinase isozymes 1 and 3 (*PDK1 and PDK3*), glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*), aldolase A (*ALDOA*), natriuretic peptide precursor B (*NPPD*), lactate dehydrogenase A (*LDHA*), hexokinase 2 (*HK2*), and hypoxia-inducible protein 2 (*HIG2*). Similar to 1% $O_2$, *o*-phenanthroline, $CoSO_4$, $CoCl_2$,

**TABLE 4**
**Correlation between Chemical Inducers and 1% $O_2$ for Hypoxia-Regulated Promoter Activity**

| Compounds | Correlation ($R$), with 1% $O_2$ | $p$ Value |
|---|---|---|
| $CoSO_4$ | 0.92 | < 0.001 |
| $CoCl_2$ (positive control) | 0.92 | < 0.001 |
| 7-Diethylamino-4-methylcoumarin | 0.10 | 0.6 |
| 7,12-Dimethylbenz(*a*)anthracence | −0.07 | 0.7 |
| Iodochlorohydroxyquinoline | 0.46 | 0.006 |
| *o*-Phenanthroline | 0.96 | < 0.001 |

*Note*. $p < 0.05$ is considered statistically significant.

and iodochlorohydroxyquinoline also stimulated several other hypoxia-regulated elements, such as those for macrophage migration inhibitory factor (*MIF*), ankyrin repeat domain 37 (*ANKRD37*), enolase 1 (*ENO1*), phosphoglycerage kinase 1 (*PGK1*), and transferrin receptor (*TFRC*). Interestingly, only 1% $O_2$ and *o*-phenanthroline induced the activity of a VEGFA long-range element, whereas the other compounds repressed the activity of the same fragment. In addition, all of the compounds, along with 1% $O_2$, decreased HIF3A promoter activity.

## DISCUSSION

In this study, we used a cell-based HRE pathway assay in qHTS format to identify twelve compounds in an NTP compound library that stimulated the expression of HIF-1α and therefore appear to act as hypoxia mimetics. For three of these compounds and the positive compound $CoCl_2$, hypoxia mimetic activities were confirmed in secondary assays of HIF-1α-dependent VEGF secretion and hypoxia-regulated promoter activity. Based on these secondary tests, we demonstrated that the hypoxia pathway responses induced by *o*-phenanthroline, iodochlorohydroxyquinoline, and $CoSO_4$ (as well as the positive control $CoCl_2$) are HIF-1α-dependent and produce hypoxia target gene promoter activation profiles similar to those induced by the standard hypoxic condition (1% $O_2$). These results suggest that the use of a pathway assay in primary screening, in combination with focused secondary assays, can effectively identify chemical mimics of hypoxia among large collections of environmental chemicals.

As the hit rate from the primary screen was relatively low, we investigated the possibility of false negative compounds. One way to estimate the false negative rate in such an assay is to examine if any of the negative compounds are closely related structurally to any of the positive compounds (i.e., a structure-activity relationship analysis approach). This approach is not definitive, however, as compounds with similar structure do not necessarily exhibit the same activity. It is also possible that compounds that are not structurally related to any of the positive compounds were false negatives as a potential consequence of compound degradation. The only way to check for compound degradation is to analytically analyze the entire library, and reorder and retest those compounds that were identified as degraded. This approach was not practical for the purpose of this study, due to time and resource constraints, but will be evaluated in a subsequent round of testing with a new compound library. Thus, we used the former approach for investigating the possibility of false negatives. We calculated the Tanimoto scores (Randic, 1997) between the positive compounds and all other compounds in the library. Assuming that all negative compounds that are structurally similar to one of the positive compounds should have been positive, which is a very conservative assumption, the estimated false negative rate for this assay is 1.2% if we adopt the traditional Tanimoto cutoff for similarity of 0.8, and 2.9% if we use the less conservative cutoff of 0.65.

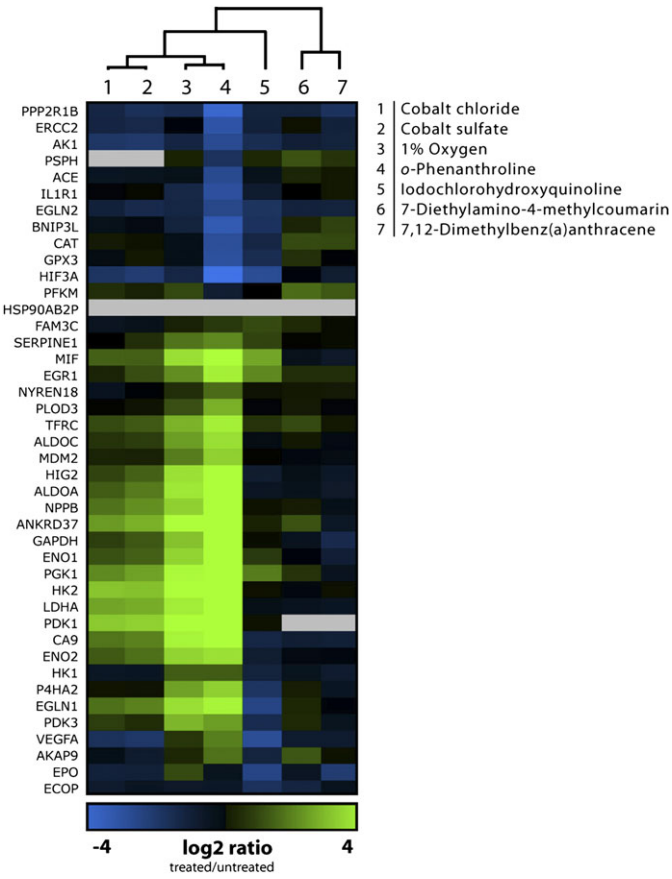


**FIG. 5.** Activity heat map of 36 hypoxia-responsive regulatory elements. The heat map illustrates hierarchical clustering for compound activation of promoter and LRE activity among six compounds plus 1% $O_2$. Each row indicates the promoter or LRE activity of a fragment in each of the compounds, with green indicating upregulated activity, blue indicating downregulated activity, black indicating no change in activity, and gray indicating no baseline or induced activity in this cell type. Luciferase values have been normalized and log transformed to reflect the relative effects of the different compounds compared with 1% $O_2$ treatment.

In Supplementary Table 2 we have listed the five confirmed active compounds as an example, and their closest structural analogs as measured by Tanimoto similarity. The closest structural analogs of *o*-phenanthroline are benzo(f)quinoline and quinoline with a Tanimoto score of 0.86. Both analogs were negative in the primary screen. As the activity of *o*-phenanthroline might be attributed to its iron chelating ability, it is not surprising that the two structural analogs do not have the same activity because neither has the structural feature necessary for iron chelation. Similarly, the closest structural analog of $CoSO_4$, another iron chelator, is sodium hydrosulfate, which does not have the ability to displace iron, and therefore, would not be expected to be active in this assay. The other active compound that can act as a chelator is iodochlorohydroxyquinoline; its closest structural analog is 8-hydroxyquinoline (Tanimoto score, 0.7), which also contains the chelation feature. We did observe a weak response with 8-hydroxyquinoline; however, this compound was considered to be a Class 4 compound because its efficacy over the concentration range tested was < 10%. This low efficacy compound may have a real effect and needs to be further investigated.

7,12-DMBA is a polycyclic aromatic hydrocarbon (PAH). PAHs, as a class, tend to give positive responses in many reporter gene assays and indeed, other PAHs in the library, such as benzo(*k*)fluoranthene, benzo(*b*)fluoranthene, and dibenz(*a*,*h*)anthracene, were active in the primary screen. However, these compounds are fluorescent, and as such, were suspected to be false positives in the primary screen. They were included in the follow-up study, however, where their activity was not confirmed. Another PAH in the collection, benzo(*e*)-pyrene, was negative in the primary screen. None of these four PAHs have substitutions on the polyaromatic ring system, whereas 7,12-DMBA has two methyl group substitutions, which may be critical for its activity in these hypoxia pathway assays. The only other PAH in the library that has methyl substitutions is 1-methylpyrene. This compound is the closest structural analog of 7,12-DMBA (Tanimoto score = 0.98; Supplementary Table 2), but it was inactive at the concentrations tested (0.59nM to 92μM) in the primary screen.

6-Methylcoumarin is the closest structural analog of 7-DEA-4-MC (Supplementary Table 2) but it has a low Tanimoto similarity score (0.65) and was inactive in the primary screen. The mechanism of action for 7-DEA-4-MC in this assay is not known, but if its activity was due to the coumarin scaffold, then 6-methylcoumarin is likely a false negative.

Of the twelve compounds identified as active in the primary screen, ten were confirmed in the primary assay and five showed activity in the VEGF secretion assay. Of these, three plus the positive control compound ($CoCl_2$) showed HIF-1α dependence and an ability to mimic hypoxia in promoter activation, so these four compounds can be confidently designated as hypoxia mimics. The two other compounds, 7-DEA-4-MC and 7,12-DMBA, will require further investigation to determine why they acted differently in the secondary screens.

Based on $EC_{50}$ values, there is discordance in the ranking order of compound potency between the HRE-*bla* assay and the VEGF secretion assay. Although the basis for this discordance is unknown, one possibility is that it reflects differences in the HRE used to drive the β-lactamase reporter gene versus the endogenous VEGF. One of the positive compounds, cobalt (Maxwell and Salnikow, 2004), had previously been suggested to be a hypoxia mimetic, which was confirmed in this study. It has also been shown that VEGF mRNA (Namiki *et al.*, 1995) and protein levels (Dai *et al.*, 2008) are significantly increased by $CoCl_2$, a finding that are consistent with hypoxia responses. We also found from our primary screening and follow-up studies that *o*-phenanthroline and iodochlorohydroxyquinoline acted as chemical hypoxia mimetics, suggesting that this approach is useful for identifying previously unsuspected hypoxia mimetics.

Pathway reporter assays such as that used here are powerful tools to identify compounds acting at any one of a number of steps in a biological process leading to changes in gene expression (Xia *et al.*, 2009). However, they rely on relatively small synthetic response elements that may not recapitulate the context in which they function in individual genes, where they often exist in the context of larger hypoxia-induced elements, hypoxia-repressed elements, and nonresponding sequences. Thus, examination of the effect of compounds identified in such reporter screens on endogenous transcriptional regulatory elements represents a useful approach to confirming genomic relevance of the primary findings. The panel approach used here provides a level of comprehensiveness intermediate between single synthetic regulatory elements and genome-wide studies of expression or factor binding.

It has been shown that hypoxia can be induced by divalent metal ions such as cobalt and nickel (Maxwell and Salnikow, 2004) and by iron chelators such as desferrioxamine (DFO) (Wang and Semenza, 1993) under normoxic conditions. The likely mechanism of HIF-1 activation by these compounds is that nickel and cobalt can substitute for the ferrous ion in regulatory dioxygenases which leads to inactivation of the enzymes (Maxwell and Salnikow, 2004). In addition to iron substitution, nickel and cobalt also bind more tightly than ferrous ions to the membrane transporter DMT-1 (divalent metal transporter 1), thereby blocking delivery of ferrous ions into cells (Maxwell and Salnikow, 2004). In this study, we found that *o*-phenanthroline, an iron chelator (Rauen *et al.*, 2007; Ryter *et al.*, 2000; Vasconcelles *et al.*, 2001), stimulated the HIF-1 signaling pathway and activated HIF-1α–dependent VEGF secretion. The profile of hypoxia-responsive promoter activities in the presence of *o*-phenanthroline is very similar to the activity profile seen under low oxygen (1% $O_2$) conditions or after treatment with cobalt, which suggests that *o*-phenanthroline may act in similar fashion to DFO by depriving cells of free ferrous ions which are essential for the activity of regulatory dioxygenases.

In comparison to cobalt and *o*-phenanthroline, iodochlorohydroxyquinoline also stimulated the HIF-1 signaling pathway and activated HIF-1α–dependent VEGF secretion, but had a slightly weaker hypoxia-regulated promoter activity correlation with 1% $O_2$. These results suggest that iodochlorohydroxyquinoline may induce hypoxia though a different mechanism(s) than cobalt and *o*-phenanthroline. Iodochlorohydroxyquinoline, also known as clioquinol, is a Cu(II)/Zn(II) specific chelator. Consistent with our findings, iodochlorohydroxyquinoline has been shown to increase functional HIF-1α protein, leading to increased expression of its target genes, VEGF and EPO, in SH-SY5Y and HepG2 cells (Choi *et al*., 2006). Iodochlorohydroxyquinoline inhibited ubiquitination of HIF-1α in a Cu(II)- and Zn(II)-dependent manner. It prevented FIH-1 (factor inhibiting HIF-1α) from hydroxylating the asparagine residue (803) of HIF-1α, which leads to the stabilization of the trans-active form of HIF-1α (Choi *et al*., 2006).

The recent National Research Council (2007) report on "Toxicology in the 21st Century" recommends a future emphasis on evaluating perturbations to "toxicity" pathways in cultured human cells. In response to this report, the NTP, the NCGC, and the U.S. Environmental Protection Agency entered into a Memorandum of Understanding in early 2008 to utilize their complementary expertise and capabilities in the research, development, validation, and translation of new and innovative test methods that characterize key steps in toxicity pathways (Collins *et al*., 2008; see also http://ntp.niehs.nih.gov/go/28213). The goal of the "Tox21" partners is to transform toxicology into a high-throughput, predictive, and mechanism-based science (Kavlock *et al*., 2008), in order to evaluate the thousands of compounds to which humans are exposed and for which there is little or no toxicological information (Judson *et al*., 2008). The results reported here, identifying compounds that are active in the hypoxia-response pathway, provide evidence that this pathway approach is both tractable and reliable for identifying compounds of potential toxicological interest.

## SUPPLEMENTARY DATA

Supplementary data are available online at http://toxsci.oxfordjournals.org/.

## FUNDING

Intramural Research Programs (NIH Interagency agreement #Y2-ES-7020-01) of the National Toxicology Program; National Institute of Environmental Health Sciences and the National Human Genome Research Institute; National Institutes of Health (NIH); and the NIH Roadmap for Medical Research Molecular Libraries Program (U54MH084681).

## ACKNOWLEDGMENTS

We thank Darryl Leja and Dr Nathan D. Trinklein for illustrations.

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